SOME USEFUL TECHNIQUES IN THE STUDY AND INTERPRETATION OF POLLEN MORPHOLOGY

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Each of the various techniques that has been utilized in the study of pollen morphology has limitations as well as advantages. It is essential, therefore, that the conclusions attained by the use of any single technique be in harmony with those obtained by other techniques. This is particularly true where pollen is subjected to more or less drastic treatments during preparation for visual examination. In all such cases evidence should be obtained regarding the effects of each treatment upon the normal form and structure of living pollen.

A large amount of research in recent years has been focused upon the visible structure of the exine of acetylated pollen. Comparatively scant attention has been devoted to the study of the intine and the protoplast. If the various layers of the pollen wall are to be accurately defined and classified, it is necessary that more be learned concerning their chemical composition and physical properties. Summations of accumulated circumstantial evidence obtained by the use of diversified techniques can provide significant clues in this connection. In so doing, generalizations should be based, not solely upon the pollen of one or two randomly selected species, but upon investigations of a wide range of representative gymnosperms and angiosperms.

Mangin attempted to do this in a brief paper published in 1889. The significance of this paper cannot be adequately appreciated without reading Mangin's other papers dealing with the differential staining, differential solubilities, and other properties of the polysaccharides that occur in plant tissues in general. He recognized, not only that the intine differs markedly from the exine, but also that it is chemically heterogeneous. He concluded that it is composed of a mixture of polysaccharides, in most cases largely of cellulose and "pectose," the cellulose being concentrated adjacent to the protoplast and disappearing in the external zone or zones and particularly in the so-called "Zwischenkörper" of Fritzsche (1837) which commonly occurs in the apertural regions of the pollen wall. It should be noted in this connection that the results of Mangin's researches on pollen were published before he obtained a sample of ruthenium red (ammoniated oxychloride of ruthenium) from Joly (1892). He (1893) considered this dye superior, in the differential staining of pectic compounds, to such basic dyes as phenosafranin, methyl blue, etc., that he utilized so extensively in his earlier investigations. However, he does not appear to have used ruthenium red at a later date in an extensive investigation of pollen.

UTILITY AND RELIABILITY OF RUTHENIUM RED

In microscopy, ruthenium red has the advantage of being soluble in water but insoluble in alcohols, anhydrous glycerin, chloroform, benzene, xylol, clove oil, and other reagents utilized in preparing sections for microscopic examination. However, in aqueous solution it has the disadvantage of deteriorating rapidly in the light at ordinary room temperatures. This difficulty can be overcome by keeping solutions in the dark in a refrigerator. I have solutions of the dye (Edward Gurr, Ltd.) that are in good condition after more than a year. A more serious difficulty occurs in attempting to make permanent mounts of stained pollen, as also of sections of plant tissues. In all cases thus far the red color disappears in time from stained parts of the preparations.

There have been two extreme views regarding the utility of ruthenium red. At one extreme are those who assume that it is specific for pectic compounds, whereas at the other extreme are individuals who have shown that it stains a variety of chemical substances and therefore conclude that it is utterly unreliable and useless in microchemical investigations. In this connection, I have made in the past extensive observations upon the staining reactions in aqueous ruthenium red of a wide range of inorganic and organic substances of known chemical composition. Tests have been made not only with naturally occurring gums and mucilages and pectic compounds and "polyuronide hemicelluloses" extracted from plant tissues, but also, through the cooperation of Professors Ernest Anderson and Karl P. Link, with fractions of these substances of known chemical composition. It seems that galacturonic and glucuronic acids when methylated, as salts, or in intimate chemical association with hexose and pentose sugars stain characteristically in aqueous ruthenium red. Where the substances themselves are soluble in water, their staining reactions may be studied by using a concentrated solution of the dye in admixture with a proportion of alcohol which inhibits or retards their rapid solution. In contrast to the general uniformity in the behavior of these naturally occurring substances, their free acids when isolated and purified are very variable in their reactions in aqueous solutions of ruthenium red. When they stain, they may give a yellow, rather than a red, coloration, and likewise may turn the color of the ruthenium solution from red to yellow. The rapidity and the intensity of staining varies in buffers of widely different pH. This raises complex questions regarding the effects of reagents in buffers upon the dye and upon the chemical composition of the substances being tested. Preliminary treatments of cells and tissues with acids, alkalies and other reagents should be avoided if possible. The most significant results are obtained when living cells or freshly cut sections of living tissues are quickly immersed in a solution of ruthenium red in distilled water or in relatively pure spring water.

Although the dye stains oxycellulose, hydrocellulose, the nucleus, protoplast, and other organic substances, as well as pectic compounds, gums, mucilages, and "polyuronide hemicelluloses," there are significant dif-

ferences in its behavior in these cases. In my investigations of cell walls of the higher plants, I have found that those naturally occurring polysaccharides which contain sugar acids (e.g., galacturonic, glucuronic) or their methylated or salt (e.g., calcium pectate) derivatives tend to stain very intensely and with extraordinary rapidity in dilute (1/5000) solutions of ruthenium red, whereas other naturally occurring organic substances, if they stain at all, do so more gradually and commonly less intensely. Of course, it should be realized in this connection that the staining may be inhibited or masked by lignification, cutinization, or suberization of cell walls. Thus, although the dye is not specific for pectic compounds and must be used with adequate precautions, its use does provide a simple and rapid method of obtaining useful clues or leads in studying the occurrence and distribution of polyuronides in plant cells and tissues. Although not conclusive by itself, it becomes increasingly so when in harmony with evidence obtained by other techniques, e.g., differential solubilities, polarized light, electron microscopy, etc.

The so-called intine of pollen commonly stains with remarkable clarity and rapidity when freshly collected pollen, viable dry pollen, dead pollen from herbarium specimens, and pollen preserved in alcohol are immersed in dilute aqueous solutions of ruthenium red. Frequently the differential staining is so rapid that it is clearly visible by the time that a mount can be made and examined under a microscope. In fact, it may sometimes be advantageous to retard the staining by using the aqueous solution in admixture with a high proportion of glycerin. Such admixtures are relatively stable when kept in the dark at ordinary refrigerator temperatures. Intense staining of the intine occurs first in the apertural region or regions of the pollen wall and subsequently in parts that subtend the exine. The exine does not stain, retaining its original greenish yellow or other colors. On the contrary the protoplast may in time develop a red coloration. The accelerated staining of thicker parts of the intine in apertural regions may be due to a higher concentration of stainable substances in such areas or to retarded penetration and diffusion of the dye through the exine of nonapertural parts. That the proportion of stainable substances varies is indicated by differences in the ultimate intensity of staining, not only as between the intines of different types of pollen, but also in different zones or layers of a single thick intine. That penetration of the dye is retarded or actually inhibited at times by the exine (or by external coatings of oily or resinous substances) is indicated by the fact that the intine of inaperturate pollen may not stain at times unless the exine is mechanically ruptured or abraded.

Ruthenium-red staining of the intine of pollen from herbarium specimens, as well as of freshly collected living pollen, provides a simple, rapid, and reliable means of studying variations in the normal form of the intine in the various taxa of the angiosperms. It is particularly useful in demonstrating variations in the form and thickness of the intine in apertural regions of pollen. Although the spectacular differences in color between the intine and exine require illustration in color, the intense red

staining of the thicker parts of the intine can be reproduced in intense black by the use of a green filter as illustrated in PLATE I.

The pollen of *Calycanthus* varies from monocolpate (*Fig. 1*) to zonaperturate or belted (*Fig. 3*) to bicolpate (*Fig. 2*) and infrequently to tricolpate. In each case the aperture or apertures are subtended by conspicuously thickened parts of the intine which stain intensely and with remarkable rapidity in dilute aqueous solutions of ruthenium red. In these pollen grains, as in those of *Illicium* (*Fig. 4*) which have three narrow grooves that extend from pole to pole,[1] the thicker parts of the intine are considerably broader than the transverse diameter of the furrows. This is in contrast to those forms of pollen (*Fig. 5*) in which the excessively thickened parts of the intine, "Zwischenkörper" of Fritzsche (1837), "oncus" of Hyde (1955), are but slightly broader than the diameter of the apertures. In the case of the Winteraceae, where the pollen occurs characteristically in tetrads, the New World representatives differ conspicuously from the Old World representatives of the family (Bailey and Nast, 1943). In the monoporate grains of the latter genera and species, the intine is somewhat thicker in the apertural part but does not protrude (*Fig. 7*), whereas in the New World representatives the protoplast protrudes and is jacketed by a thick coating of intensely staining intine (*Fig. 8*). In a majority of the angiosperms the thickenings of the intine subtend the apertures of varying form, size, and number, but this is not invariably the case, as has been shown by Dressler (1957) in the Euphorbiaceae. For example, in the tricolporate pollen of *Pedilanthus* (*Fig. 6*) there are six riblike thickenings which extend nearly from pole to pole. These thickenings of the intine are lateral to the elongated colpae, rather than directly beneath them.

OCCURRENCE AND DISTRIBUTION OF CELLULOSE IN POLLEN

Although the intense ruthenium-red staining of the outer part of the intine, particularly in apertural regions of pollen, is not necessarily indicative of pectic composition, it suggests, in correlation with the isotropy of this part in polarized light, plasticity during pollen tube emergence, expansion and contraction during wetting and drying, and solubility in cold 4% sodium hydroxide and other reagents which do not dissolve cellulose, that the outer part of the intine commonly contains a large proportion of uronide polysaccharides in its chemical composition.

As regards the inner part of the intine, the differing conclusions attained by Mühlethaler (1953) and Sitte (1953) by the use of electron microscopy raise an important question regarding the occurrence of cellulose in the inner part of the intine adjacent to the protoplast as hypothesized by Mangin (1893). In diversified representatives of both gymnosperms and angiosperms this part of the intine, although staining more or less

[1] For illustrations compare Wodehouse (1935), page 336, *Fig. 92*, or Erdtman (1952), page 256, *Fig. C*.

intensely in ruthenium red, exhibits anisotropy in polarized light. Furthermore, it stains a characteristic blue when pollen grains are immersed directly in chloro-iodide of zinc or in 65% sulphuric acid following staining in iodine. Where the intine is very tenuous and obscured by the exine, the cellulosic part may be retained and rendered clearly visible by removing the protoplast and the noncellulosic constituents of the intine in 3% sodium hydroxide at 56° C. (*Figs. 9–11*).

Not only is the inner part of the intine insoluble in reagents which do not dissolve cellulose, but also it is removed by standard solvents of cellulose, e.g., 72% sulphuric acid, cuprammonium reagents, as well as during prolonged acetylation of pollen. Furthermore, its microfibrillar composition can be revealed by electron microscopy as demonstrated by Sitte (1953). In addition, it should be noted that when the noncellulosic constituents of the intine are completely removed the cellulose-containing inner layer no longer stains rapidly and intensely in ruthenium red.

In general (with the exception of certain unusual forms of pollen, e.g., *Eupomatia*), the cellulose-containing inner layer of the intine of both gymnosperms and angiosperms is tenuous and of relatively uniform thickness. This is in contrast to the striking variations in the thickness of the outer part of the intine that occur so frequently in various taxa of the higher plants. During the disruption of pollen walls due to differences in the contraction or expansion of the protoplast and wall layers, the cellulosic layer of the intine commonly tends to remain adjacent to the protoplast.

SOLUBILITY OF THE EXINE IN MONOETHANOLAMINE

The exine of pollen, like the cuticle of plants, is generally considered to be relatively inactive chemically since it persists for such prolonged periods in geological strata and dissolves only after prolonged drastic treatments. Much to my surprise, therefore, I have found that exines of freshly collected living pollen at anthesis (e.g., of such gymnosperms as *Taxus*, *Tsuga*, *Pseudotsuga*, *Pseudolarix*, and *Pinus* and of such angiosperms as *Liriodendron*, *Magnolia*, *Asimina*, *Taraxacum*, *Coreopsis*, *Ostrya*, *Populus*, and *Calycanthus*) dissolve in three hours or less when immersed in monoethanolamine at a temperature of 97° C. The only exceptions that I have encountered thus far in a preliminary investigation are pollen of *Ephedra* and *Pinus strobus* from old herbarium specimens and of *Eupomatia* preserved for a long period in F.A.A. fixative (formalin-acetic acid-alcohol). The fact that the exine of freshly collected pollen of *Pinus* dissolves suggests that changes occur under certain conditions of prolonged drying which inhibit solubility. Unfortunately no freshly collected pollen of *Ephedra* and *Eupomatia* is available, as yet, for such comparative purposes. In the case of *Liriodendron*, it is possible, by adequately controlling temperature and time of treatment, to remove the thick exine (*Fig. 12*) leaving the protoplast, the layers of the intine and oil globules but slightly modified visually (*Fig. 13*). Of course,

this raises an important question, viz., whether dissolving the exine of a large amount of pollen in monoethanolamine might yield a solute capable of separation and analysis. Such analyses might afford significant clues regarding the chemical composition of the exine and deserve to be more intensively investigated.

DISCUSSION

Summations of evidence obtainable by the application of diversified techniques indicate that the cellulosic part of the intine of both gymnosperms and angiosperms occurs in a comparatively narrow zone adjacent to the protoplast. The anisotropic cellulose occurs, however, in association with an isotropic polyuronide (or mixture of polyuronides and polysaccharides) which stains rapidly and intensely in dilute aqueous solutions of ruthenium red and which is readily removable by reagents which do not dissolve cellulose. The outer part of the intine, particularly in thickenings related to apertures, contains little, if any, cellulose and is composed of a polyuronide (or a mixture of polyuronides and polysaccharides) which stains and dissolves as does the material associated with the cellulose in the innermost part of the intine. The noncellulosic constituent in thicker parts of the intine (related to apertures in the exine) is plastic (i.e., easily penetrated or pushed aside during emergence of the pollen tube) and contracts and swells, with corresponding invagination and evagination, during drying and rewetting of living pollen. The consistency in the occurrence and behavior of this part of the intine in a wide range of taxa suggests that it serves two important functions, (1) to protect the protoplast in apertural parts of the wall and (2) to facilitate emergence of the pollen tube. Professor A. Orville Dahl and I plan to discuss this aspect of the intine in greater detail in a subsequent joint paper. We are also correlating evidence obtained by phase and electron microscopy and other diversified techniques in detailed studies of specific forms of pollen. The question of a revised terminology for wall layers, e.g., whether the use of intine should be restricted to the cellulosic layer and exintine or mesine used in referring to material that intervenes between the cellulosic layer and the exine, had best be deferred until comprehensive investigations of a wide range of representative pollen forms have been completed.

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EXPLANATION OF PLATES

PLATE I

Pollen differentially stained in aqueous ruthenium red and photographed with a green filter at a magnification of 770. Figs. 1–3, 6, freshly collected living pollen; 5, 8, pollen from herbarium specimens; 4, pollen preserved in 70% alcohol; 7, pollen preserved in FAA fixative. Figs. 1–3, *Calycanthus* sp. (cultivated, Cambridge, Mass.): 1, monocolpate grain viewed in optical section parallel to long axis of colpus; 2, dicolpate grain viewed in optical section parallel to the long axis of the colpus; 3, zonaperturate grain viewed in diagonal optical section. Fig. 4, *Illicium floridanum* Ellis (cultivated, Henry Foundation, Gladwyne, Pa.), tricolpate grain, polar view. Fig. 5, *Nouhuysia arfakensis* (Gibbs) Steenis (*Kostermans 2198* [A]), triporate grain. Fig. 6, *Pedilanthus* sp. (*Dressler*), tricolporate grain, polar view. Fig. 7, *Zygogynum Baillonii* Tiegh. (*Buchholz*, New Caledonia, 1947), each grain of the tetrad with a single more or less circular aperture. Fig. 8, *Drimys granadensis* L. f. var. *mexicana* (DC.) A. C. Smith (*Hinton 14441* [GH]), characteristic tetrad of New World section *Wintera*, each grain of the tetrad with a single aperture and protruding protoplast jacketed by a thick coating of stainable intine.

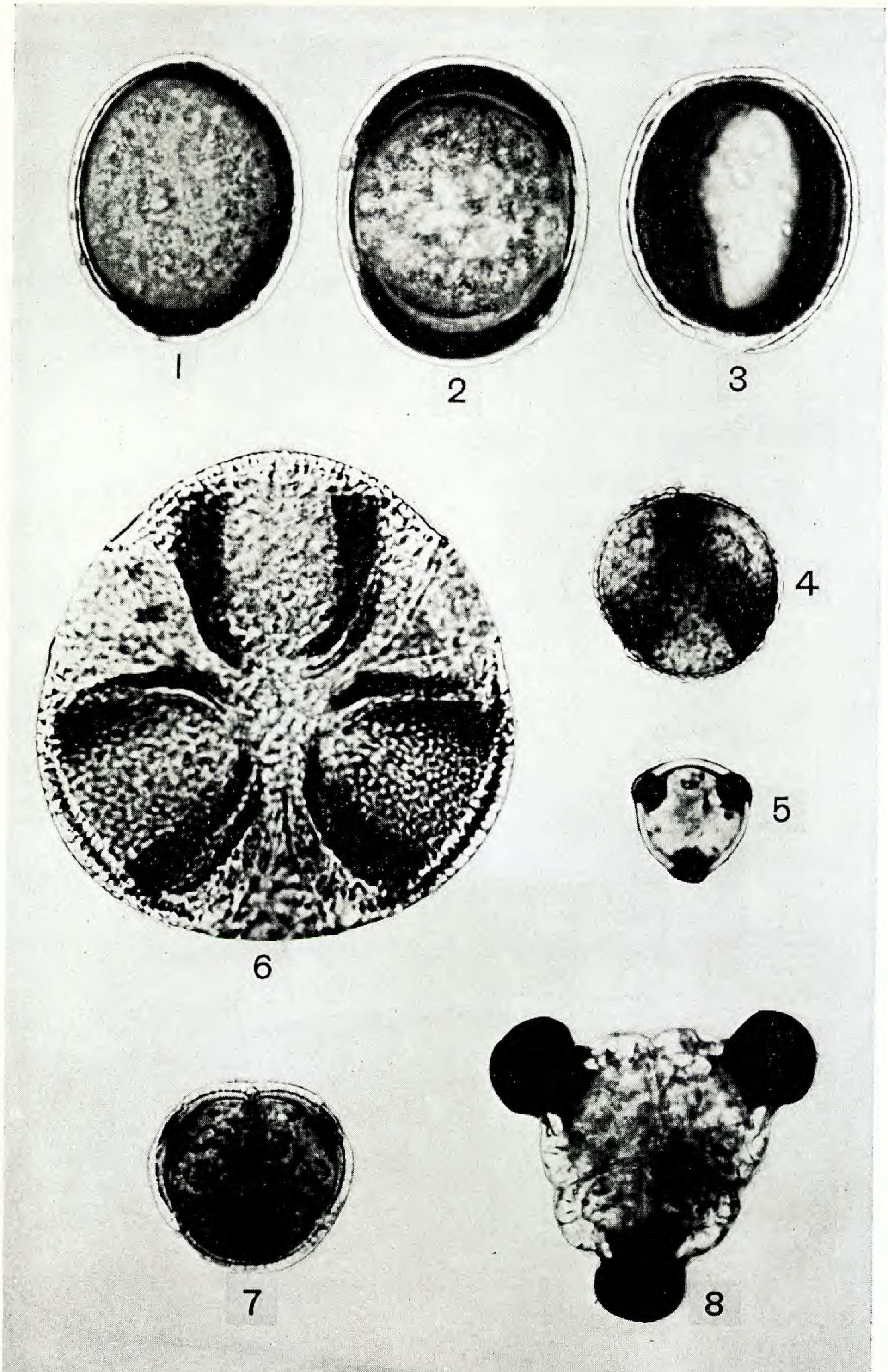
PLATE II

Figs. 9, 10, *Liriodendron tulipifera* L. (cultivated, Arnold Arboretum): 9, pollen after treatment in 3% NaOH at a temperature of 56° C. which removes the protoplast and the noncellulosic constituents of the intine — when im-

mersed in chloro-iodide of zinc the contracted cellulosic residue of the intine stains a deep blue, in striking contrast to the greenish-yellow color of the exine, × 1130; 10, characteristic anisotropy of two cellulosic residues in polarized light, × 400. FIG. 11, *Pinus strobus* L. (Norwell, Massachusetts), freshly collected living pollen following the same treatment as in FIG. 9, × 1130.

PLATE III

FIGS. 12, 13, *Liriodendron tulipifera* (cultivated, Arnold Arboretum): 12, freshly collected living pollen in a mixture of water and glycerine, viewed in optical section parallel to the long axis of the colpus, showing coarsely warty exine and conspicuous thickening of the intine in the apertural region, × 1130; 13, pollen grain after a brief treatment in monoethanolamine at a temperature of 97° C. — exine has been removed leaving the intine, protoplast, and oil globules in place, × 1130.



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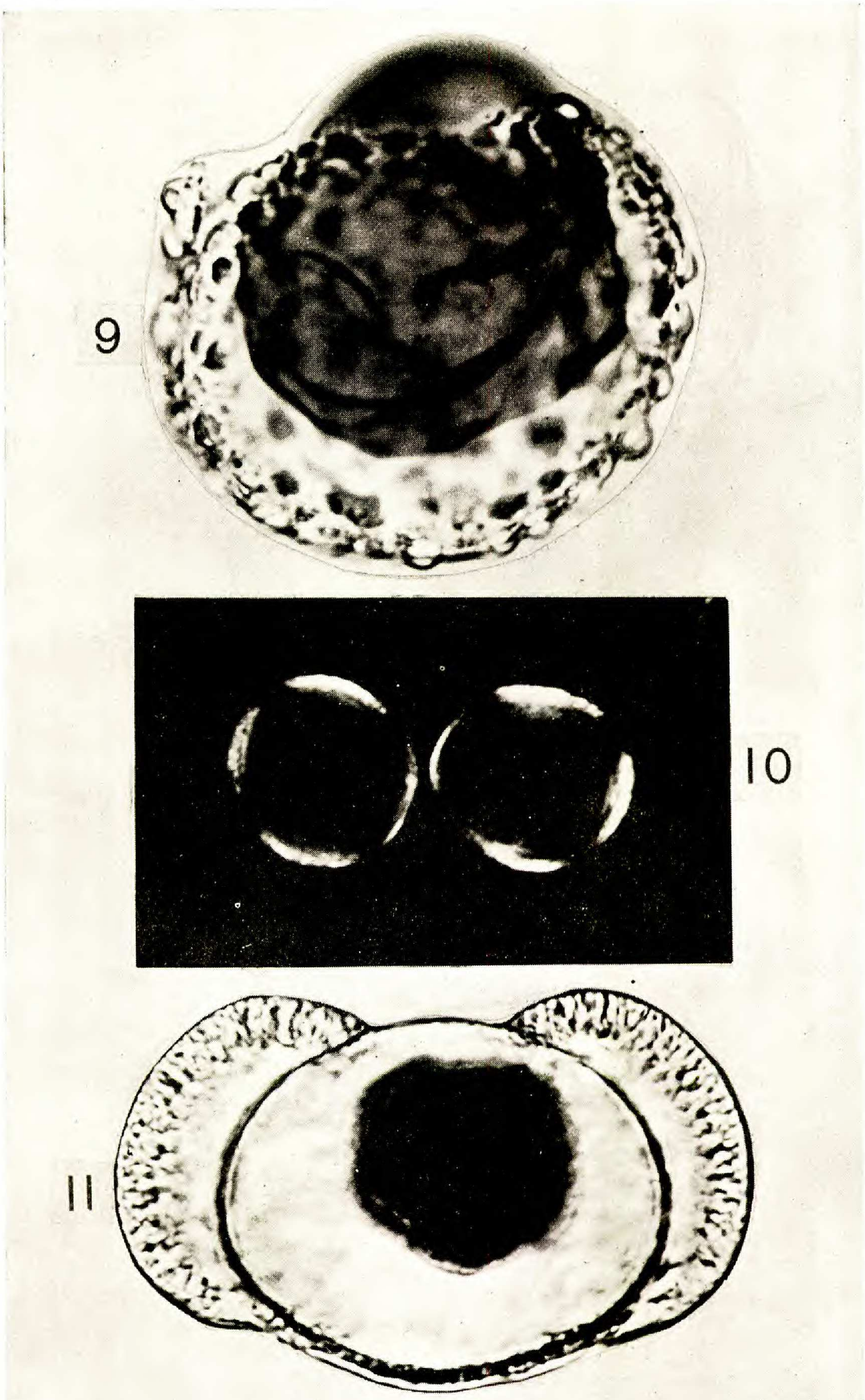

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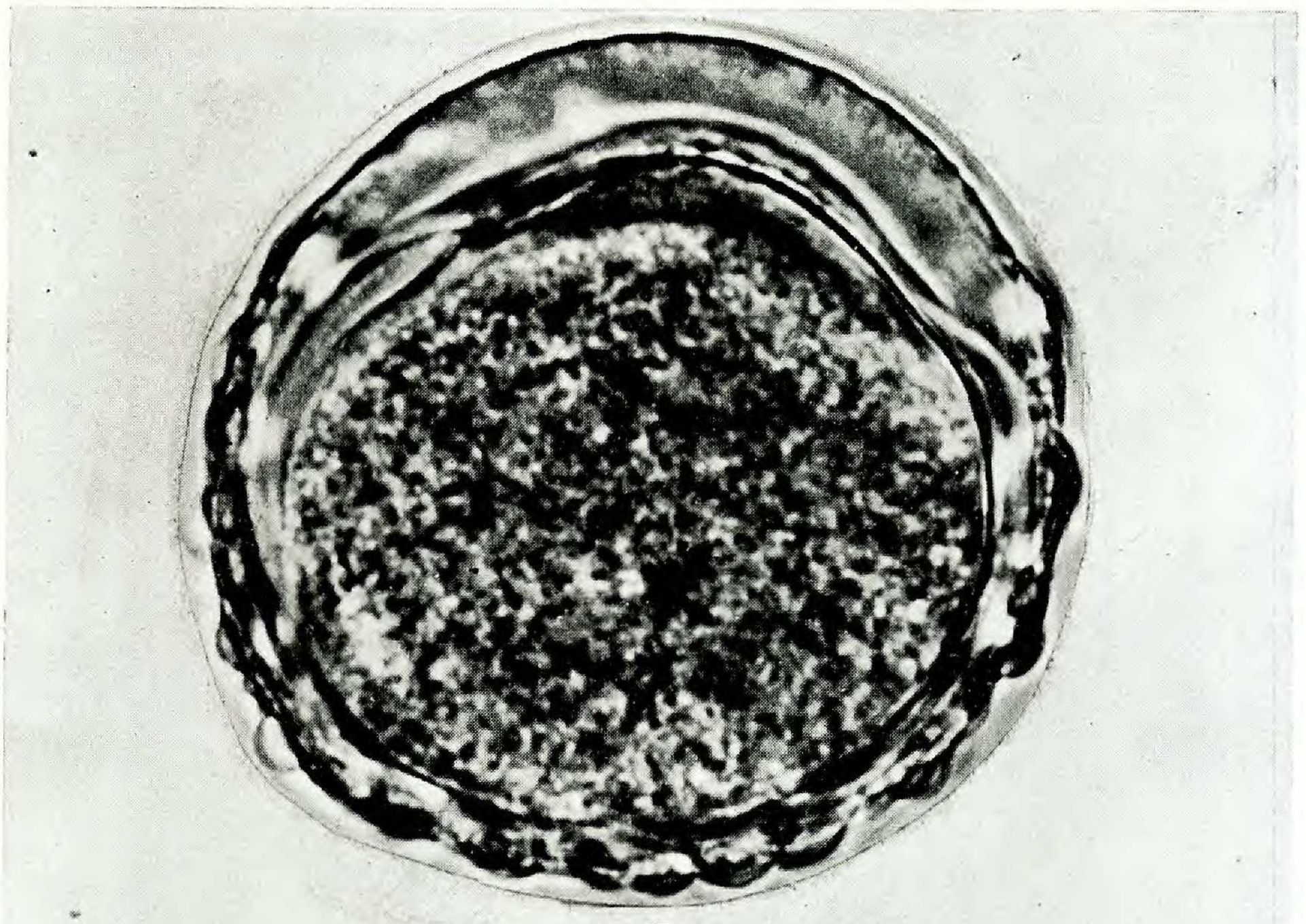

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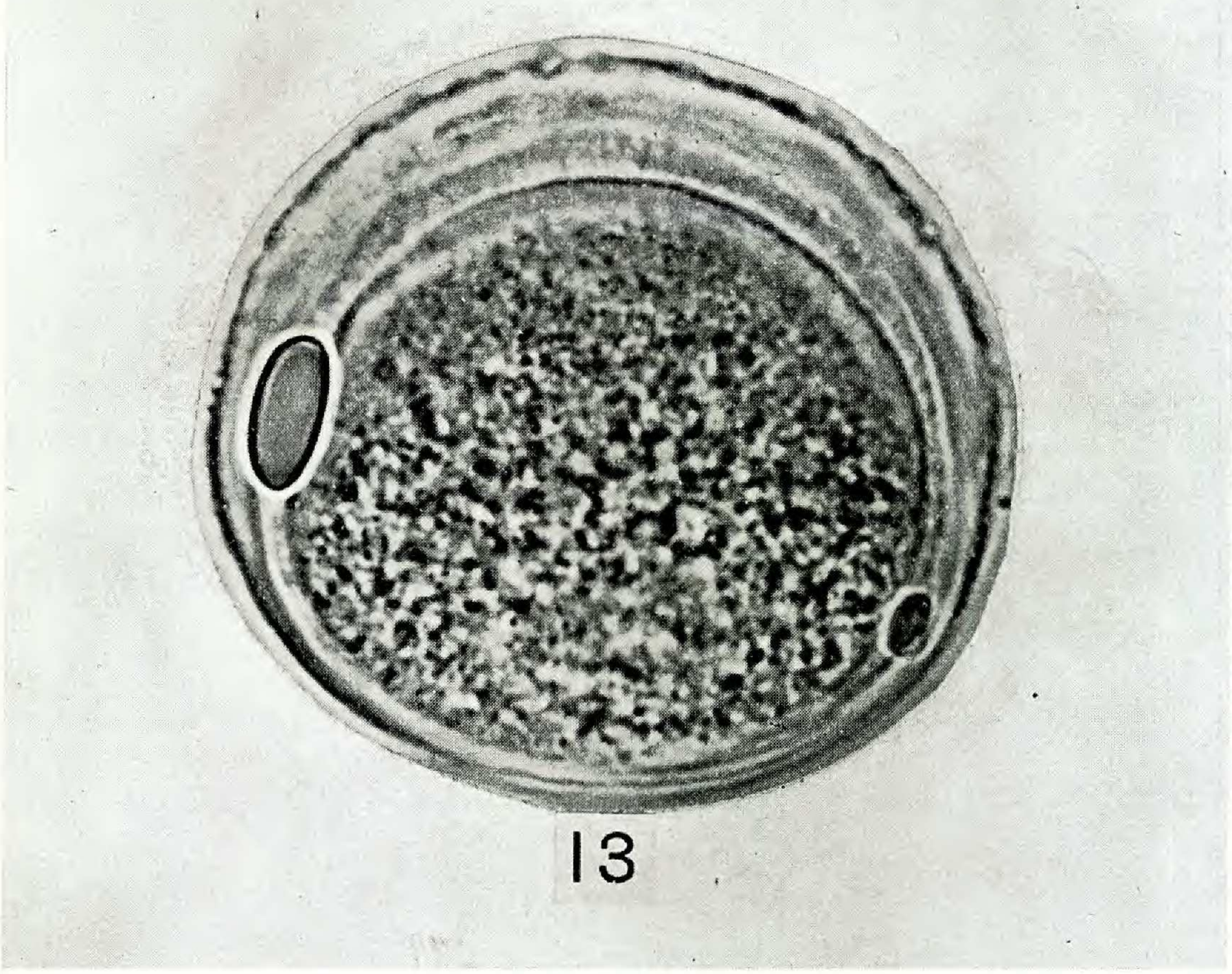

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